HLA antibodies are associated with deterioration of kidney allograft function irrespective of donor specificity

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Abstract: BACKGROUND Donor-specific antibodies are associated with high immunological risk and poor allograft outcome. Risk and clinical relevance of non-donor-specific HLA antibodies is less clear. METHODS A retrospective single-center study was conducted in all patients receiving a first kidney transplant at the University hospital of Zürich between 01/2006 and 02/2015. Patients were stratified into 3 groups having either no HLA antibodies at all (NoAB), HLA antibodies with donor specificity (DSA) and HLA antibodies without donor specificity (NonDSA). Allograft outcome was assessed using the slope of the estimated glomerular filtration rate (eGFR slope) starting at 12 months after transplantation. RESULTS During a median follow-up of 1808 days HLA antibodies were detected in 106 of 238 eligible patients (44%). Out of these, 73 patients (69%) had DSA and 33 patients (31%) had NonDSA only. Medium-term allograft function, as determined by eGFR slope over three years, improved in patients with NoAB (months 12-48: +0.7 ml/min/1.73 m^2) but deteriorated significantly in patients with both DSA (months 12-48: -1.5 ml/min per1.73 m^2/year, p = 0.015) and NonDSA (months 12-48: -1.8 ml/min per1.73 m^2/year, p = 0.03) as compared to the group with NoAB. CONCLUSION Both, donor-specific and non-donor-specific HLA antibodies are associated with medium-term kidney allograft dysfunction as compared to patients with no HLA antibodies.

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Research article

HLA antibodies are associated with deterioration of kidney allograft function irrespective of donor specificity

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ABSTRACT

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Conclusion: Both, donor-specific and non-donor-specific HLA antibodies are associated with medium-term kidney allograft dysfunction as compared to patients with no HLA antibodies.

1. Introduction

Current short-term kidney allograft survival is outstanding, however, long-term allograft survival remains suboptimal. Chronic allograft injury, associated with progressive fibrosis and declining renal function, is the major cause of long-term allograft loss. Both immunologic and non-immunologic processes contribute to allograft injury, yet the underlying mechanisms are not completely understood [1]. Early identification of patients at risk of graft loss would be desirable, to allow early intervention and to predict graft survival. Detection of donor-specific HLA antibodies (DSA) has been a major focus of study and DSA are an established biomarker associated with deterioration in allograft function [2,3]. Development of DSA is associated with antibody-mediated rejection (ABMR) as most important cause for late graft loss [1,2,4–7]. Patients with pre-formed DSA are known to be at increased risk of early graft failure [8]. Importantly, *de novo* DSA develop in 11% of transplant patients by year 1 post-transplantation and 30% by year 10 post-transplantation, and are also associated with poorer transplant outcomes [9,10]. Thus, routine screening for occurrence of DSA by single antigen beads such as the Luminex single antigen bead (SAB) assay is recommended [11,12]. With the currently available Luminex SAB assays for detection of DSA, a considerable number of patients also show non-donor-specific HLA antibodies (NonDSA). The clinical relevance of NonDSA is less clear. The seminal paper by Opelz, et al. [13] showed that even in HLA-identical siblings, the presence of panel-reactive antibodies (PRA) against HLA antigens before transplantation was associated with reduced graft survival beyond the first year, suggesting that NonDSA play a role in late allograft failure. Likewise, further studies observed a negative impact of pre-transplant NonDSA on graft survival [13–15]. Similar observations were also reported for detection of NonDSA post-transplantation [16,17]. In contrast, other groups have reported, that pre-transplant sensitization *per se* is not

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Nomenclature

AB	HLA antibodies
ABMR	Antibody-mediated rejection
AKI	Acute kidney injury
CI	Confidence interval
CNI	Calcineurin inhibitor
CKD	Chronic kidney disease
CKD-EPI	Chronic kidney disease epidemiology collaboration creatinine equation
DD	Deceased donor
DSA	Donor-specific HLA antibodies
eGFR	estimated glomerular filtration rate
LD	Living donor
MFI	Mean fluorescence intensity
NoAB	No HLA antibodies
NonDSA	Non-donor-specific HLA antibodies
PRA	Panel reactive antibodies
SAB	Single antigen bead
TCMR	T-cell-mediated rejection

associated with increased immunological risk of graft loss except if DSA are present [15,18,19]. Hence, findings regarding this clinically highly relevant question of immunological risk associated with NonDSA on allograft outcome are controversial.

In this study, we aim to investigate the impact of pre- and post-transplant NonDSA on allograft function. Most prior studies either compared graft survival between different groups stratified by their pre-transplantation antibody profile [13–15,18] or applied a cross-sectional design comparing graft loss or glomerular filtration rate at a given time point depending on presence or absence of HLA antibodies[16,17]. Here, we analyzed the longitudinal eGFR slope as surrogate marker for long-term kidney function [20–23] in relation to the presence or absence of HLA antibodies as detected by Luminex assay.

2. Patients and Methods

2.1. Study design and patient population

In this single-center retrospective cohort study, all adult kidney allograft recipients receiving their first transplant between January 2006 and February 2015 at the University hospital of Zürich were included with a maximum period of observation for kidney function till February 2016. Recipients younger than 18 years of age, or multi-organ transplantations, as well as patients with prior kidney transplantation and ABO incompatible kidney transplantations were excluded. The study was approved by the local Ethics committee of Zürich (Basec number: 2017–00500) and performed in adherence to the declaration of Helsinki and the Declaration of Istanbul on Organ-Trafficking and Transplant-Tourism.

2.2. Patient follow-up and immunosuppression

All kidney transplant recipients are seen at least annually at our center. In case of graft dysfunction or other complications, patients are referred earlier for follow-up. Immunosuppressive regimens follow our internal guidelines: Induction therapy with T-cell depleting agents (Thymoglobulin®) is given to patients with pre-formed DSA, interleukin 2 receptor antagonists (basiliximab) to recipients with non-donor-specific HLA antibodies (NonDSA) or no HLA antibodies at all (NoAB). Pre-formed DSA are accepted only if mean fluorescence intensity (MFI) < 10′000 and only if not multiple DSA > 1000 are present and only if crossmatch negative. Maintenance immunosuppression consists of a calcineurin inhibitor (cyclosporine or tacrolimus) and an anti-proliferative drug (mycophenolate mofetil). Target trough levels at 6, 12 and 24 months are 100–160 ng/ml, 80–120 ng/ml, 50–80 ng/ml and 7–10 ng/ml, 6–8 ng/ml, 4–6 ng/ml, for cyclosporine and tacrolimus, respectively. Steroids are generally withdrawn 6 months post-transplantation. Kidney biopsies are performed as indication biopsies in patients who develop *de novo* DSA and/or have signs of allograft function deterioration (i.e. increasing creatinine and/or increasing proteinuria). If antibody-mediated rejection (ABMR) is present, patients are treated with a steroid bolus, a switch to a tacrolimus-based triple-immunosuppression and, depending on acuity and severity, with combinations of IVIG, rituximab, immune adsorption or plasmapheresis. Detection of *de novo* DSA without functional deterioration and without biopsy proven ABMR does not entail intensification of immunosuppression.

2.3. Stratification into immunological risk groups according to Luminex results

HLA antibody monitoring using Luminex mix assay (pooled antigen panel beads with different class I or II HLA antigens, LABScreen Mixed Class I and II antibody screening kit, OneLambda Canoga Park, CA, USA) is routinely performed at least once a year or more frequently in case of a deterioration of graft function or development of proteinuria. If the Luminex mix assay is positive, an additional Luminex single antigen bead (SAB) assay is performed at least once to specifically test for HLA-A/B/C and HLA-DRB1, DR51/52/53, DQA1/DQB1 and DPA1/DPB1 antibodies (LabScreen Single Antigen Beads, OneLambda Inc.). Donor and recipients were all typed for HLA-A, -B, and -DR and from 2012 onwards also for -DQ). Calculated MFI values are normalized against the internal negative control and the negative serum control. If an allele specific antibody against the donor is discovered in the single antigen Luminex screening assay, it is labeled as a DSA.

Patients were stratified into immunological risk groups considering all available pre- and post-transplant anti-HLA-Antibodies detected by Luminex. The following immunological risk groups were defined: patients without HLA antibodies (NoAB) or with HLA antibodies (AB). The AB group was further stratified into patients with HLA antibodies with donor specificity (DSA) and patients with HLA antibodies without donor specificity (NonDSA). To ensure correct patient stratification, patients with inconclusive data by Luminex assay were excluded from the analysis: i.e.: positive Luminex mix without verification with Luminex SAB, antibody MFI levels < 1000 (i.e. below the cutoff for a positive Luminex assay according to the Swiss Organ Procurement System), no Luminex results within 12 months of the most recent follow-up visit, and positive DQ antibody without DQ locus typing.

2.4. Data analysis and primary outcome

All relevant baseline and outcome data were collected retrospectively from electronic medical records. eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration creatinine equation (CKD-EPI) [24]. The primary outcome was the

eGFR slope in the medium post-transplantation period (three-year slope, months 12–48) between the different immunological risk groups. The eGFR slope was calculated by linear regression using all available creatinine measurements. A minimum of three creatinine measurements per time interval were required for the eGFR slope calculation. eGFR slopes were determined starting at 12 months post-transplantation (baseline; margin ± 2 months) for different, cumulative time intervals post-transplantation: months 12–24, 12–36, 12–48, and 12–60, respectively (Suppl. Fig. 1A–E). Patients with severe acute kidney injury (AKI) requiring dialysis were completely excluded from the analysis, as creatinine values during AKI would introduce a relevant bias in slope calculation. Proteinuria was assessed by protein/creatinine ratio in random spot urine measurements.

2.5. Statistics

eGFR slopes were calculated by linear regression using R statistical package. Descriptive statistics of the variables analyzed are presented as mean ± 95 confidence interval (CI). Independent groups were compared using Mann Whitney-*U* test in case of non-Gaussian data distribution or students *T*-test if data passed normality tests (Pearson omnibus). Non-categorical data was compared with chi-square test. For all tests, a p-value of < 0.05 was considered as statistically significant. Statistical analysis and figures were done with Graph Pad Prism software.

3. Results

3.1. Study population and immunologic risk groups

From January 2006 to February 2015 a total of 738 patients received a kidney transplantation at the University hospital of Zürich. Patient characteristics are presented in Tables 1. Of 416 patients meeting the inclusion criteria 178 patients (42.7%) were excluded because of insufficient data for eGFR slope calculation (Fig. 1A). This exclusion ensured uniform groups and robust data with respect to description of eGFR slope. Median follow-up time of the remaining 238 patients was 1808 days, i.e. 60 months (25. percentile 1199 days, 75.percentile 2528 days).

HLA antibodies (AB) were detected in 106 (44%) patients. Out of these 33 (31%) patients had only non-donor-specific HLA antibodies (NonDSA), whereas 73 (69%) patients had in addition donor-specific HLA antibodies (DSA). Of note, all of these patients had DSA and NonDSA, but are referred to the group with DSA throughout the manuscript. (Fig. 1B). Detection of *de novo* DSA occurred at a median time of 1078 days, i.e. 35 months post-transplantation (95% CI 1063–1614 days) and detection of *de novo* NonDSA occurred at a median time of 743 days, i.e. 24 months post-transplantation (95% CI 624–1472 days).

With respect to the burden of HLA antibodies, there was no difference between the two groups in the number of HLA antibodies per individual patient ($p = 0.07$, data not shown). However, specificity of HLA antibodies did significantly differ between the two groups: patients with DSA showed predominance for class II HLA antibodies ($p < 0.0001$), while patients with NonDSA only, showed predominance for class I HLA antibodies ($p = 0.0001$) (Fig. 1C + D).

3.2. Presence of HLA antibodies is associated with a progressive deterioration of graft function

One-year kidney allograft function, reflecting the starting point of the eGFR slope trajectory, was not different among patients with AB and those with NoAB (Fig. 2A). Over the following periods of three years (months 12–48) the median eGFR slope was + 0.7 ml/min/1.73 m2/year in patients with NoAB. In contrast, in patients

Table 1A
Patient characteristics of the two groups with and without HLA antibodies.

	No HLA antibodies (NoAB)	HLA antibodies (AB)
Number, n	132	106
Preformed antibodies, n	–	52
De novo antibodies, n	–	54
Male gender, n (%)	95 (72)	58 (55)
Female gender, n (%)	37 (28)	48 (45)
Age at transplantation, mean years, (95% CI)	53 (51; 55)	50 (47;52)
Follow up time, median days, (min; max)	2047 (355; 3405)	1781 (346; 3645)
Detrimental Outcome (%)	6 (0.05)	6 (0.06)
Graf failure	2	1
Death with functioning Graft	4	5
Biopsies (%)	5 (4)	65 (61)
Immune reaction	1	31
TCMR	1	13*
ABMR	0	19*
other	4	34
No biopsy (unknown)	127	41
Kidney transplant, n		
Living donor transplant (%)	54 (41)	44 (42)
Deceased donor transplant (%)	78 (59)	62 (58)
Donor age deceased donor, years (95% CI)	53 (50–57)	47 (43–52)
Primary renal disease, n		
Glomerulonephritis (%)	29 (22)	27 (25)
Polycystic disease (%)	36 (27)	16 (15)
Urologic disease (%)	6 (5)	6 (6)
Hypertension (%)	17 (13)	6 (6)
Diabetic Nephropathy (%)	8 (6)	4(4)
Other (%)	36 (27)	47 (44)

*One biopsy with TCMR and ABMR.

Table 1B
Groups of patients with HLA antibodies: DSA vs nonDSA.

	Group HLA Antibodies (AB)	
	DSA	NonDSA
Number, n	73	33
Preformed antibodies, n (%)	34 (47)	18 (55)
De novo antibodies, n (%)	39 (53)	15 (45)
Male gender, n (%)	35 (48)	23 (70)
Female gender, n (%)	38 (52)	10 (30)
Age at transplantation, mean years, (95 CI)	50 (47;53)	48 (43;53)
Follow up time, median days, (min; max)	1540 (446; 3546)	1829 (348; 3645)
Time to de novo antibodies, days (95% CI)	1078 (1063–1614)	743 (624–1472)
Detrimental Outcome (%)	5 (0.07)	1 (0.03)
Graft failure	1	0
Death with functioning graft	4	1
Biopsies (%)	48 (6)	17 (51)
Immune reaction	24	7
TCMR	6*	7
ABMR	19*	0
other	24	10
No biopsy (unknown)	25	16
Kidney transplant		
Living donor transplant (%)	33 (45)	11 (33)
Deceased donor transplant (%)	40 (55)	22 (66)
Donor age deceased donor, age (95% CI)	48 (42–53)	47 (40–55)
Primary renal disease		
Glomerulonephritis (%)	15 (21)	12 (36)
Polycystic disease (%)	13 (18)	3 (10)
Urologic disease (%)	4 (5)	2 (6)
Hypertension (%)	4 (5)	2 (6)
Diabetic Nephropathy (%)	2(3)	2 (6)
Other (%)	35 (48)	12 (36)

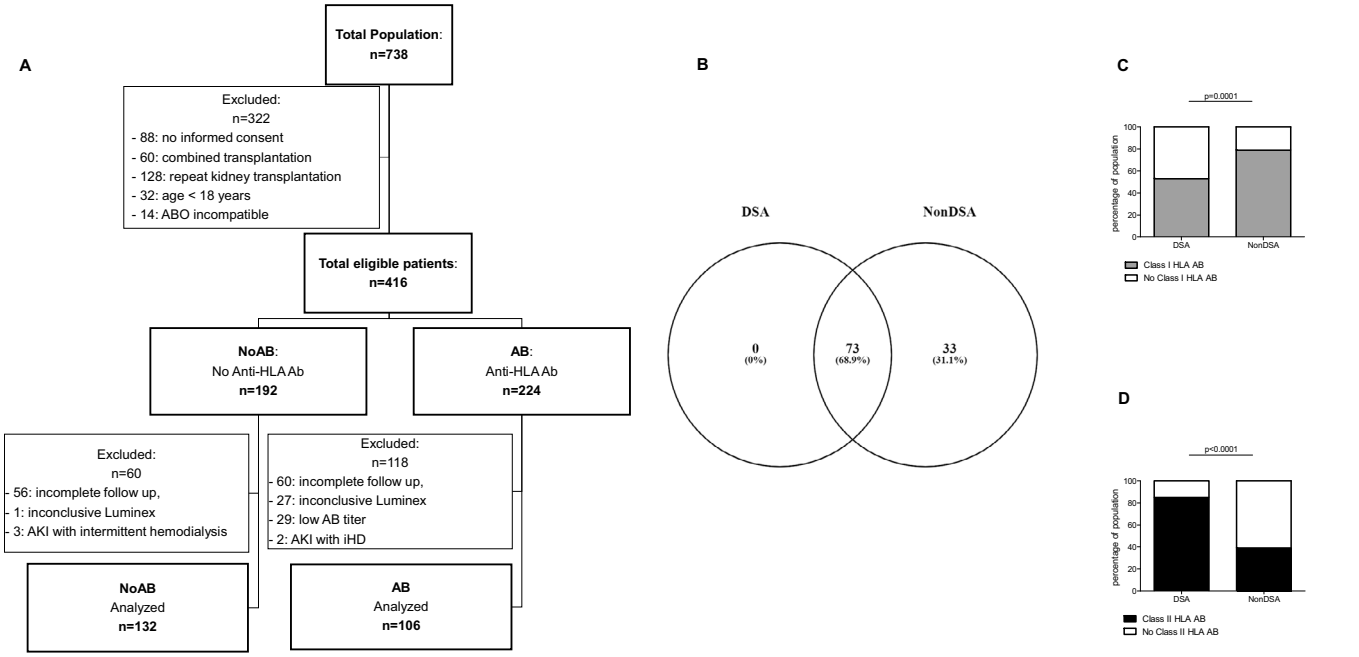

Fig. 1. Study flow chart A) Study flow chart with patient stratification into two main immunological risk groups: NoAB and AB. B) Further stratification of patients with AB: All 73 patients with DSA also have NonDSA. C) Percentage of Class I and D) Class II HLA antibodies in patients with DSA and NonDSA. Comparison by Fishers exact test.

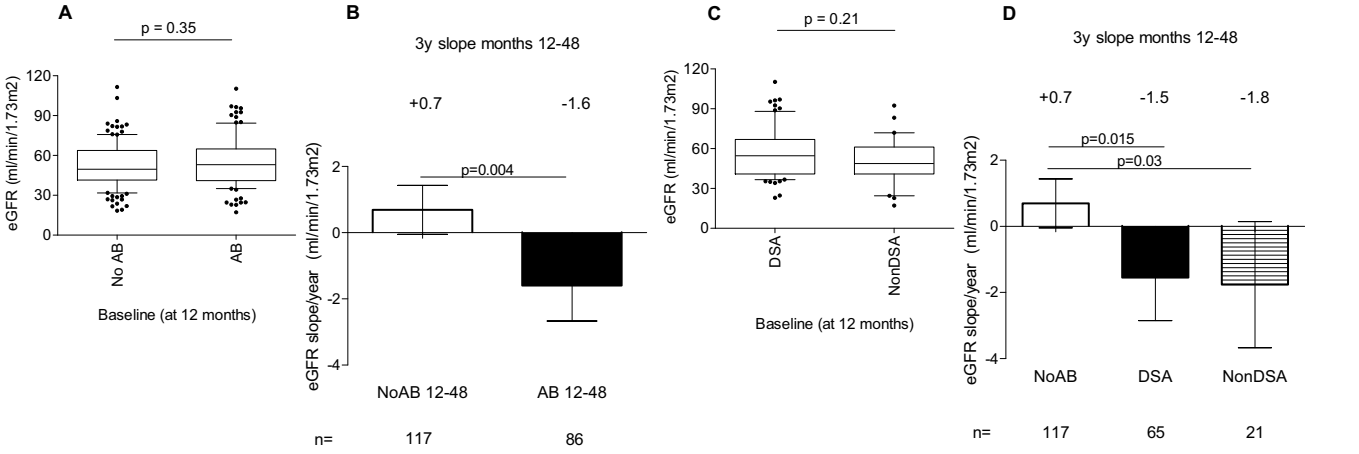

Fig. 2. Baseline eGFR and three-years eGFR slope in patients with different immunological risk groups A) Baseline eGFR at 12 months in patients with NoAB as compared to patients with AB. B) Three-years eGFR slope (months 12–48) in patients with NoAB as compared to patients with AB. C) Baseline eGFR at 12 months in patients with DSA as compared to patients with NonDSA and D) Three-years eGFR slope (months 12–48) in patients with DSA and NonDSA as compared to patients with NoAB. Box plots showing median and 10 and 90. percentile. eGFR slope data is shown as mean ±CI. Group comparison by Mann Whitney *U* test.

with AB, the three-year eGFR slope was negative with −1.6 ml/min/1.73 m2/year (p = 0.006 as compared to NoAB) (Fig. 2B). This results in a difference in eGFR slope over three years of 2.3 ml/min/1.73 m2/year between patients with antibodies versus without antibodies (AB vs. NoAB). Consistently, eGFR slope was also positive for all the other time intervals both for living as well as deceased allografts with NoAB (Suppl. Fig. 2A) and negative at all time intervals for patients with AB (Suppl. Fig. 2B).

Important to note, that differences in eGFR slope between the different immunological risk groups were not due to different levels of immunosuppression (Suppl. Fig. 3A and B). Furthermore, despite significant differences in eGFR slopes between the two groups, levels of proteinuria were not significantly different between patients with AB and those with NoAB (Suppl. Fig. 3C). Additionally, graft failure rate over the whole observation period was not significantly different between the groups (1 graft failure in AB group, 2 graft failures in NoAB group) (Table 1A).

3.3. Both, DSA and NonDSA negatively impact graft function, however with a later onset in patients with NonDSA

Subgroup analysis of eGFR in patients with AB stratified in those with DSA and those with NonDSA confirmed a similar allograft function at one year post-transplantation (Fig. 2C). Over the next three years a significantly negative eGFR slope occurred for patients with DSA as compared to patients with NoAB (eGFR slope months 12–48 after transplantation: −1.5 ml/min/1.73 m2, p = 0.015). Similarly, patients with NonDSA also showed a significantly negative eGFR slope over three years as compared to patients with NoAB (eGFR slope months 12–48: −1.8 ml/min/1.7

3 m2/year, p = 0.03) (Fig. 2D). These results remained unchanged even when including all patients with DSA and NonDSA below and above MFI threshold 1000 (*data not shown*). The comparison of the courses in eGFR changes in yearly time intervals between patients with NoAB and patients with DSA or NonDSA, showed development of a negative eGFR slope in patients with DSA already in the early post-transplantation period, while eGFR slope turned significantly negative later in patients with NonDSA, i.e. in the medium-term post-transplantation period (Suppl. Fig. 4). Of note, eGFR slope was not significantly different in patients with either pre-formed or *de novo* DSA or NonDSA, respectively (Suppl. Fig 5A and B). When further differentiating patients with DSA and NonDSA in those with persistent and intermittently detectable antibody, results were highly consistent in those with persistently detectable DSA and NonDSA, but more variable in the other groups (Suppl. Fig 6A–F).

4. Discussion

Detection of HLA antibodies is associated with deterioration of renal allograft function as measured by negative eGFR slope over three years, which reflects the medium follow-up time post-transplantation. Such functional deterioration is observed irrespective of donor specificity of HLA antibodies as compared to patients with no detectable HLA antibodies. Our study is novel in that we have examined the association between presence of HLA antibodies and eGFR slope, while others have focused on graft failure in relation to sensitization status. Of note, despite a significant difference in eGFR slope between different immunological risk groups, there was no difference in graft failure rates over the whole observational period in our cohort. This suggests that eGFR slope is more sensitive in detecting changes in allograft function already at early time points, while graft loss being a late endpoint. The calculation of eGFR slope to predict the trajectory of kidney disease progression has initially been described by Mitch et al. [20] and has recently been re-adopted and validated as surrogate end point for progression to kidney failure in CKD trials[21,23] and graft failure after ABMR[21,23,25] Hence, the National Kidney Foundation workgroup for establishing early surrogate endpoints of kidney disease progression recently showed, that a difference in treatment effects between two treatments of 0.74 ml/min per 1.73 m2/year mean difference in total eGFR slope over 3 years was associated with a 97.5% probability of a clinically relevant benefit[21]. Referring to our observation of a 2.3 ml/min per 1.73 m2/year between group difference of eGFR slope over 3 years for patients with NoAB as compared to those with AB, we therefore hypothesize, that our observation of functional deterioration in patients with AB, irrespective of donor specificity, is not only statistically, but also clinically relevant.

While a group from the Mayo clinic reported an average eGFR slope of −1 ml/min per 1.73 m2/year [26] for the whole cohort of kidney transplant recipients between year 1 and 5 post-transplantation, they did not differentiate between different immunologic risk groups. Yet they state that the majority of patients showed stable or improving graft function, with only a subgroup of grafts showing functional deterioration. Consistently, we observed in our cohort for patients with NoAB an improvement of allograft function as reflected by a positive eGFR slope. Such functional improvement in the absence of immunologic tissue injury could be explained by the kidney's functional plasticity (adaptive hyperfiltration), as it is observed after unilateral nephrectomy in kidney donors [27,28]. Alternatively, it might be a consequence of the reduction in calcineurin inhibitor dose over time when allograft function remains stable. In contrast to this low immunological risk group, we observed a negative slope in the medium post-transplantation period for both groups with AB, i.e. DSA and NonDSA. While the association of DSA with functional allograft deterioration is well known in the literature [2,7,9,29], the role of NonDSA is debated [13–18]. While some previous studies with very long follow-up have suggested a negative impact of NonDSA on 10-year [13] or longer [17] allograft survival, more recent studies investigating 5-year allograft survival rates and biopsy proven ABMR could not detect an effect of NonDSA [15,18]. We hypothesize, that the analysis of graft function by eGFR slope as done in our study, might detect more subtle changes induced by non-donor-specific immune responses leading to graft loss in the very long-term only, which are unlikely to be captured with 5-year allograft survival rates and might explain the controversial results. According to our results, both pre-transplant and *de novo* occurrence of NonDSA, especially if persistently detectable by Luminex, are associated with allograft function deterioration.

From a pathophysiological point of view and based on previous reports of earlier appearance of NonDSA as compared to DSA [17], we speculate that detection of NonDSA may reflect inflammatory tissue injury. Such responses, irrespective of donor specificity, might be associated with late allograft functional deterioration, corresponding to chronic allograft nephropathy. Recent studies investigating the pathophysiology of chronic allograft failure have indeed shown an important role for natural antibodies, i.e. antibodies reacting to multiple distinct, self and non-self antigens, generated in the absence of DSA and occurring as a reaction to ischemia–reperfusion injury [30]. Hence, in this context, NonDSA might represent a nonspecific marker of ongoing injury and healing and may be a precursor of later development of donor-specific alloreactivity contributing to late graft fibrosis and dysfunction, a scenario previously shown in animal work [31].

Our study design reflects the real clinical setting and identifies associations, however the results do not allow any interpretation in terms of causality. A major strength of our study is the granularity of data with respect to HLA antibody screening and eGFR slope analysis, which has recently been evaluated as a surrogate marker for early detection of end stage renal disease [4,21,23,25]. Consistent annual screening of all the transplant patients in our cohort was performed over a long follow-up period with HLA antibody detection by Luminex mix assay and consecutive Luminex SAB assay if indicated. Integration of these data with longitudinal functional follow-up and calculation of individual eGFR slopes helps to better understand the natural course of individual allograft function in the presence or absence of an immunological response, and might permit early detection of subtle changes and identification of 'at risk' grafts for late-stage graft loss. Application of strict criteria regarding patient stratification and eGFR slope calculation ensured homogenous subgroups and robust data sets, yet it meant excluding a high percentage of patients. Hence, a bias towards patient selection cannot be excluded, which is a limitation of our study. Furthermore, misclassification of some patients is possible as mixed Luminex assay were not always associated with the higher sensitivity single antigen detection; neither C, DPA, DPB loci or high resolution typing were regularly performed. Also, we did not routinely search for non-HLA antibodies. Moreover, we did not capture data on cardiovascular risk factorsthat might also affect eGFR. Lastly, protocol biopsies are not performed in our center. Therefore, a correlation of immunological and functional data with biopsy results was not possible and unfortunately, the group of special interest, i.e. patients in the NonDSA group with functional deterioration were not biopsied (Suppl. Fig. 7A + B). Hence, the pathophysiological link remains speculative.

In conclusion, we show that renal allograft function, as assessed by eGFR slope, declines similarly in the medium-term after transplantation in patients with HLA antibodies, irrespective of donor specificity. In contrast, allograft function tends to improve in the

medium-term post-transplantation in patients with no HLA antibodies. Our results therefore contribute to the understanding of immunological processes after kidney transplantation and support the clinical relevance of NonDSA with respect to late deterioration in allograft function.

5. Authorship contributions

SvM was involved in designing the study, data collection, statistical analysis and manuscript preparation. PC was involved in designing the study and manuscript preparation. RvB was involved in data collection and data analysis. TM contributed to the study design and the finalization of the manuscript. All authors approved the final manuscript.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Dr. von Moos receives a travel funding from Astellas. Dr. Cippà reports a travel funding from Vifor Pharma. Dr. Mueller and Rob van Breemen have no disclosures.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.humimm.2020.10.010.

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